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Haemoglobin adducts as biomarkers of exposure to tobacco-related nitrosamines

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Abstract
A sensitive gas chromatography/mass spectrometry (GC/MS) method was developed to measure nitrosamine–haemoglobin adducts (HPB-Hb) (4-hydroxy-3-pyridinyl-1-butanone) at trace levels in red blood cells of smoking and non-smoking mothers and their newborn babies. GC/MS methods with chemical ionization (CI) of methane reagent gas in both positive and negative ion mode as well as electron ionization (EI) were studied to determine differences in sensitivity among the various ionization methods. Detection limits using both positive and negative chemical ionization modes were found to be 30 fmol HPB, whereas detection using electron impact modes yielded a detection limit of 80 fmol HBP. In order to apply the various methods of detection to tobacco-exposed samples from human populations, we characterized adduct levels in maternal as well as paired fetal samples obtained from mothers exposed to tobacco smoke during pregnancy. Maternal samples were characterized using serum cotinine levels and were classified as non-smokers, passively smoke-exposed women, less than one pack per day smokers and greater than one pack per day smokers. Paired maternal and fetal blood samples were obtained at delivery for qualitative and qualitative analysis of nitrosamine adducts. Comparative derivatization of HPB released under alkaline hydrolysis conditions was performed using O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and 2,3,4,5,6-pentafluorobenzoylchloride (PFBC). Both negative CI and positive CI modes of analysis were compared to the more widely accepted EI modes of mass spectrometric analysis. These results suggest that both NICI and PICI modes of detection offer a greater sensitivity of adduct characterization when compared with EI ionization techniques and that either NICI or PICI modes are preferably applicable towards the detection of human biomarker assessment of tobacco-related nitrosamines.

Keywords: *Biomarkers, exposure, nitrosoamine–haemoglobin adducts, tobacco, smoking, GC/MS*

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Introduction

Smoking cigarettes during pregnancy has been shown to increase the risk of numerous adverse pregnancy outcomes, including low birth weight, preterm delivery, miscarriage, ectopic (tubal) pregnancy, infant death, low Apgar scores and early childhood illness (respiratory illness, asthma). Some biological mechanisms that have been clinically

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confirmed linking cigarette smoke to fetal health, include an association between nicotine, decreased placental blood flow and an increase in fetal heart rate. Evidence suggests that a dose–response relationship exists between cigarette consumption, especially during the third trimester and neonatal birth weight (Chiolero et al. 2005, Hammoud et al. 2005, Bryan & Hindmarsh 2006, Obed & Patience 2006, Ingvarsson et al. 2007). The prevalence of smoking during pregnancy has been estimated at between 15 and 30% of all pregnant women with the percentages varying slightly dependent on the source of the data used and with State-to-State variations (Kentucky generally ranks among the highest States in percentage of women smokers). Recently the Centers for Disease Control (CDC) reported that the incidence of State-specific smoking prevalence among US adults varied widely ranging from a low of 14.2% Utah to a high of 30.8% in Kentucky. In addition, animal studies have clearly shown that exposure of pregnant rats and mice to tobacco smoke during gestation results in the reduced number of live births as well as an increase in teratogenic events in the offspring (Sardas et al. 1995, Haustein 1999, Nelson et al. 1999a, b).

Cigarette smoke is known to contain more than 40 known or putative carcinogens (Hoffmann et al. 1991). Many of these compounds are not only carcinogenic when applied directly to animals, but also have considerable teratogenicity to pregnant rats and mice (Reckzeh et al. 1975). Two classes of chemicals found in tobacco which appeared to be most widely recognized as being both carcinogenic as well as toxic include the tobacco-specific nitrosamines as well as the polycyclic aromatic hydrocarbons (LaVoie et al. 1987, Rodriguez et al. 1999, Joseph et al. 2005). Both of these classes of compounds have been clearly demonstrated to give rise to animal tumours of a variety of types upon exposure or inhalation and it is widely regarded that these classes of compounds potentially play a role in the aetiology of human cancers caused by exposure to tobacco (Haustein 1999, Nelson 1999b). As a result, it is of great importance to carry out studies on the determination of human exposure assessments to the various carcinogens found in tobacco and also to develop risk-assessment protocols that may predict an individual's overall risk of developing tobacco-related disease.

Previous studies on tobacco-exposure assessment have relied heavily on the use of serum or urinary cotinine measurements as sole indicators of exposure to tobacco (Akiyama et al. 2006, Dukic et al. 2007, Mansi et al. 2007). Cotinine is the primary metabolite of the tobacco constituent nicotine and is widely regarded as one of the classic biomarkers of tobacco exposure (Joseph et al. 2005, Akiyama et al. 2006, Sorensen et al. 2007). Numerous studies have demonstrated that levels of cotinine detected in populations correspond reasonably well with recent tobacco exposure and these assays have been reliably applied to the assessment of smokers as well as non-smokers. Although cotinine is widely regarded as the ideal indicator of tobacco exposure, there are several drawbacks when using cotinine assessments as the only indicator for long-term, chronic exposure assessments. Primarily, one of the problems with the use of cotinine is in the fact that its half-life is relatively short, approximately 24 h (Haufroid & Lison 1998). As a result, measurement of cotinine will reflect only the most recent exposures to tobacco and will not give any indication of past smoking histories or exposures to tobacco. This is particularly the case when one is dealing with maternal smoking habits during pregnancy, especially when one is trying to ascertain long-term habits rather than short-term exposure. Ideally, the most appropriate biomarker for studies such as these would be a biological marker of tobacco exposure

which takes into account not only acute recent exposure, but also includes longer exposures that have occurred in the past. In addition, in studies of maternal and fetal exposure to tobacco smoke during pregnancy, it is quite common for women to have long and protracted labours, resulting in relatively low cotinine levels when compared to the actual smoking histories over the preceding several weeks. As a result, alternative biological markers of exposure assessment have been proposed and many have been applied to the application of tobacco assessments (Hecht 2003, Okoli et al. 2007, Sorensen et al. 2007). A variety of tobacco-exposure biomarkers have been extensively studied in previous reports and numerous biological markers of exposure have been proposed (Bartsch et al. 1990, Hecht et al. 1991, Smith et al. 1992, Scherer et al. 2000). These biomarkers have consisted of characterization of specific protein adducts of tobacco carcinogens as well as detection of urinary metabolites of specific components of tobacco (Acosta et al. 2004, Bernert et al. 2005, Tulunay et al. 2005, Matt et al. 2006). Each of these biomarkers has been shown to be effective in assessing human exposures to tobacco-related carcinogens and has been successfully applied in human exposure assessment models. However, limited studies have directly compared differing modes of analytical assessment.

The protein haemoglobin found in the red cell provides an ideal surrogate marker for exposure assessments studies. Haemoglobin contains numerous nucleophilic sites, including valine, lysine, histidine, and sulfhydryl amino acid residues among others that have sufficient reactivity to bind to reactive electrophiles formed by metabolic activation of carcinogens (Bartsch et al. 1990, Tannenbaum 1990, Hecht et al. 1991, Ehrenberg & Tornqvist 1992, Ehrenberg et al. 1996, Osterman-Golkar & Bond 1996). In addition, haemoglobin has a biological half-life in circulation in the red cell of 120 days, therefore ensuring a long-term biological assessment of potential exposures (Angerer et al. 2007). Haemoglobin has been used effectively as a biological marker for numerous environmental chemicals, including vinyl monomers, butadiene, polycyclic aromatic hydrocarbons and other chemicals that are known to be hazardous to human populations (Richter & Branner 2002, Wu et al. 2004, Airoldi et al. 2005, Ogawa et al. 2006, Sabbioni et al. 2006, Scherer et al. 2007). Application of haemoglobin as a biomarker of exposure assessment is widely accepted as a useful tool in the biomonitoring of environmental contaminants and continues to yield valuable information regarding risk assessment and population exposure assessments to a variety of differing compounds (Bader et al. 1994, Falter et al. 1994, Osterman-Golkar & Bond 1996, Scherer et al. 2000).

Of the thousands of chemicals found in tobacco, the tobacco-related nitrosamines as well as the polycyclic aromatic hydrocarbons comprise what is regarded as the most toxic and carcinogenic class of compounds. The tobacco-specific nitrosamines NNN (N'-nitrosonornicotine) and NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) are the most potent carcinogens in tobacco smoke and smokeless tobacco products. These nitrosamines induce tumours of the lung, liver, nasal cavity and pancreas in the rat and are implicated in potential human carcinogenesis caused by tobacco exposure (LaVoie et al. 1987, Hoffmann et al. 1991, 1994). NNK is metabolically activated to a reactive species that binds covalently to haemoglobin and to DNA and as a result these covalent modifications can be accessed by cleavage and the resulting released product characterized using mass spectrometric tools (Hecht et al. 1991). The current study assesses a variety of techniques for characterizing both qualitatively and quantitatively the formation of tobacco-related nitrosamines in both maternal and fetal cord blood

obtained from smokers and non-smokers. This series of investigations will lead to an improvement in the methods of detection for the various tobacco-related compound, and will also lead to a better understanding of maternal exposure during pregnancy and the potential effects that smoking has on fetal exposures to various carcinogens. A reaction scheme of NNK metabolism pathways by microsomal enzymes and adduct formation is shown in Figure 1.

NNK requires metabolic activation to form both methylated and pyridyloxobutylated DNA adducts and primarily haemoglobin cysteine adducts. Microsomal enzymes initiate a hydroxylation reaction at the methyl carbon adjacent to N-nitroso group resulting in α-hydroxylation derivative. The hydroxylated NNK is then metabolized to a pyridyl-oxobutyl-diazonium ion (I) which is the reactive electrophilic metabolite. This NNK-derived diazonium ion can react with any physiological macromolecules which has available neucleophilic sites, e.g. DNA, haemoglobin, etc. Confirmation of this pathway has been demonstrated with evidence demonstrating that inhibition of α-hydroxylation results in decreased levels of DNA and protein adducts, as well as significant reduction in tumour incidence and multiplicity.

Several reports have focused on the tobacco-specific nitrosamine–DNA adducts in smokers and non-smokers in animal models using gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS-MS) techniques. However, very few studies have been carried out investigating the formation of these biomarkers in a variety of classifications of smoking populations, including passively smoke-exposed individuals, as well as maternal–fetal pairs (Hecht et al. 1991). The majority of the methods that have been previously applied for the characterization of nitrosamine–haemoglobin adducts involved a multistep process of extraction and chromatographic isolations prior to analysis utilizing GC/MS (Hecht et al. 1991, Falter et al. 1994, Atawodi et al. 1998). These methods for the most part were extremely time-consuming, and had limited practicality in clinical situations where hundreds of samples were being run simultaneously. The goal of our study was

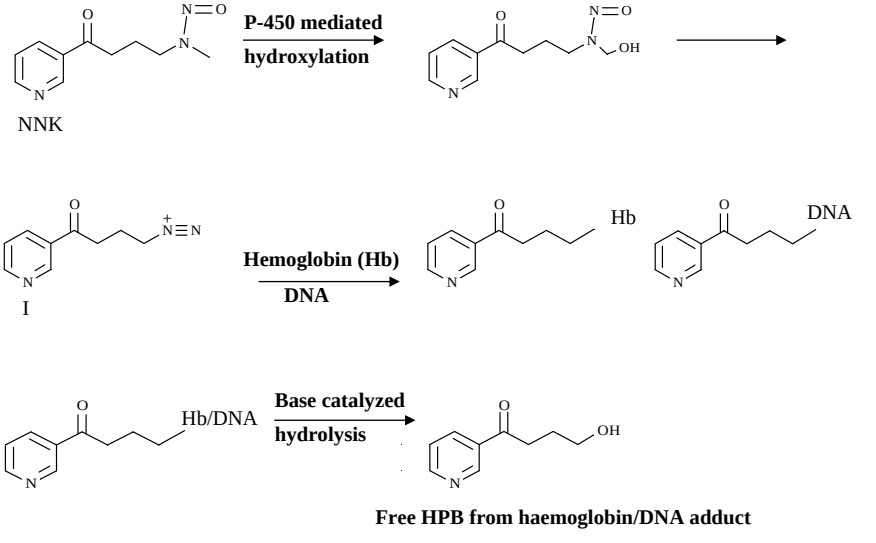

Figure 1. Metabolism of the tobacco-specific nitrosamine NNK to reactive species that can for both methylated and pyridyloxobutylate adducts of DNA as well as haemoglobin sulfhydryl adducts.

to develop a new sensitive method which would involve simple extraction techniques without a chromatographic clean-up prior to GC/MS operation.

Materials and methods

4-Hydroxy-1-(3-pyridyl)-1-butanone (HPB) and deuterated (3,3,4,4-D_4)HPB were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Pierce Biotechnology Inc. (Rockford, IL, USA) and 2,3,4,5,6-pentafluorobenzoylchloride (PFBC) was purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). All additional chemical and solvents used throughout the experiments were of the highest grade commercially available.

Blood samples

Matched maternal and fetal cord blood samples were obtained from Norton Suburban Hospital in Louisville, Kentucky under the University of Louisville approved IRB protocol. All samples were obtained in 10 ml purple top EDTA-containing vacutainer tubes and refrigerated immediately upon acquisition. Whole blood was centrifuged at 3000*g* for 30 min to generate packed red cells. After centrifugation, the plasma was removed, and the pack cells washed twice with isotonic saline to remove residual nitrosamines and other contaminants that may have been present in plasma or loosely bound to the red cell membranes. After washing the packed red cells, the cells were lysed by the addition of the 3 volumes of ice cold double-distilled water and the haemoglobin preparation resulting from the lysing of the red cells was centrifuged at 10 000*g* for 30 min to remove cellular debris. The resulting haemoglobin solution was gently pipetted into separate tubes (1–2 ml) and stored at -70°C until processing for haemoglobin–nitrosamine adducts. Aliquots of 100 µl haemoglobin were removed for spectrophotometric determination of haemoglobin concentrations using absorbancy of oxyhaemoglobin at 415 nm and the molar extinction coefficient of 125 mm^{-1}. An additional aliquot of 10 µl was removed from each sample for the assessment of cotinine concentrations using immunoassay kits (Cozart Bioscience, Alsingdon, Oxfordshire, UK). Maternal haemoglobin samples were separated into non-smokers (less than 5 ng/ml cotinine), passively smoke-exposed individuals (5–15 ng/ml cotinine), less than one pack per day smokers (15–50 ng/ml cotinine) and greater than one pack per day smokers (>50 ng/ml cotinine). All samples were stored under nitrogen at -70°C until processing of haemoglobin for adducts.

Sample preparation HPB-haemoglobin adduct assay by GC/MS

One hundred microlitres of lysed haemoglobin solution were placed in a 7 ml borosilicate glass tube with screw cap telflon liner; 750 µl HPLC grade water and 150 µl 1 N NaOH were added to the haemoglobin solution; 10 µl of 2.5 µg/ml (3,3,4,4-D_4)HPB was added as the internal standard (IS). The solution was incubated in air at 50°C for 2 h. Following incubation, the haemoglobin solution was acidified by addition of 225 µl 1 N HCl, and washed by the addition of 2 ml dichloromethane and 2 ml *n*-hexane. The washed solution was neutralized by addition of 75 µl 1 N NaOH. After

washing, the samples were extracted twice with 2 ml dichloromethane, the extracts combined and dried under a gentle stream of nitrogen gas, and stored at −20°C.

Stock solutions of HPB and (3,3,4,4-D_4)HPB were prepared in acetonitrile at concentrations of 1.25, 5.0, 12.5, 50.0, 125.0 and 500.0 ng/ml. Trimethylsilyl derivatization of HPB and (3,3,4,4-D_4)HPB were prepared by the addition of 10 µl BSTFA and 30 µl acetonitrile to samples followed by incubation in air for 1 h at 60°C. HPB-pentafluorobenzoate derivatization was performed by addition of 1 µl PFBC in 49 µl *n*-hexane:triethylamine (50:1) followed by a 1 h incubation in air at 60°C. The derivatized samples were transferred to GS/MS sample vials and were diluted to a total volume of 100 µl with acetonitrile. Samples were analysed in triplicate using both negative and positive chemical ionization modes as well as electron impact ionization modes and reported as pmoles HPB/g Hb ± SEM.

GC/MS methods

A HP 6890 GC coupled to HP 5973 MS was interfaced with the Hewlett-Packard ChemStation software package for data acquisition and analysis. GC conditions were optimized with a temperature programming to attain the highest sensitivity and resolution. Helium carrier gas was used with a flow rate 1.3 ml/min. Gas chromatographic conditions included injection of samples using splitless injection mode with an injection port temperature at 280°C. A GC capillary column consisting of a DB-15MS with dimensions 15 m × 0.25 mm id × 0.25 µm film thickness (J&W Scientific, Folsom, CA, USA). Samples were eluted through the GC column using a temperature program consisting of an initial temperature of 50°C for 1 min, followed by a ramp increase in temperature to 230°C at 20°C/min, followed by a final isothermal temperature at 230°C for 5 min.

Positive ion chemical ionization

Full scan (m/z 50-450) positive ion chemical ionization (PICI) mass spectra data were acquired to characterize derivatized HPB and (3,3,4,4-D_4)HPB. (3,3,4,4-D_4)HPB was used as internal standard (IS). Mass spectrometer conditions consisted of electron energy 221 eV, source temperature 250°C, emission current 237 µA, and electron multiplier voltage 1718 V. Quantitation of trimethylsilyl derivative of HPB by BSTFA was performed using selected ion monitoring (SIM) at m/z 238 and 242 for analyte and IS, respectively. Pentafluorobenzoate derivatized HPB by PFBC was also characterized using SIM at m/z 360 and 364 for analyte and IS, respectively (Table 1).

Negative ion chemical ionization

Full scan (m/z 50-450) negative ion chemical ionization (NICI) mass spectra data were acquired to identify derivatized HPB and (3,3,4,4-D_4)HPB. Mass spectrometer conditions consisted of electron energy 193 eV, source temperature 150°C, emission current 49 µA and electron multiplier voltage 2996 V. Quantitation of the trimethylsilyl derivative of HPB was performed using selected ion monitoring (SIM) at m/z 237 and 247 for analyte and IS, respectively. The HPB-pentafluorobenzoate derivative was also characterized using SIM at m/z 359 and 363 for analyte and IS, respectively (Table 2).

Table 1. Quantification of HPB (4-hydroxy-1-(3-pyridyl)-1-butanone) derivatized with BSTFA (O-bis(trimethylsilyl)-trifluoroacetamide) in maternal and fetal cord blood samples using positive ion chemical ionization mass spectrometry.

Smoking status	Maternal (pmol HPB/g Hb)	Maternal (pmol HPB/g Hb)	Fetal (pmol HPB/g Hb)	Fetal (pmol HPB/g Hb)
Non-smokers ($n=45$)	0.52±0.22	0.42±0.13	0.25±0.13	0.13±0.06
Passively exposed ($n=26$)	2.16±0.98	1.86±0.35	1.31±0.75	0.64±0.35
<1 pack per day smokers ($n=83$)	5.24±1.35	4.77±0.68	2.16±0.86	1.52±0.74
>1 pack per day smokers ($n=91$)	12.65±2.41	16.32±1.16	6.52±1.63	7.86±1.55

Electron impact ionization

Full spectrum (m/z 50-450) electron impact ionization (EI) mass spectra data were acquired to characterize derivatized HPB and (3,3,4,4-D_4)HPB. Electron impact ionization conditions included electron energy 70 eV, source temperature 230°C, emission current 35 μA, and electron multiplier voltage 1859 V. Quantification of derivatized HPB by BSTFA was performed using SIM at m/z 222 and 226 for analyte and IS, respectively (Table 3).

Results

PICI, NICI and EI techniques were investigated to develop a sensitive GC/MS procedure for the assessment of HPB (4-hydroxy-1-(3-pyridyl)-1-butanone) released from haemoglobin adducts upon base catalyzed hydrolysis. Derivatization techniques using BSTFA (N,O-bis(trimethylsilyl)-trifluoroacetamide) and PFBC (2,3,4,5,6-pentafluorobenzoylchloride) were also compared to improve the detection of HPB.

PICI is a soft ionization technique which generates a strong parent molecular ion along with relatively few fragmentation ions. In PICI, the reagent gas is ionized by collision with emitted electrons from the electron source. The reagent gas ions then react chemically with the gas molecules obtained from the sample during volatilization to form positively charged molecular ion containing an extra proton $[M+H]^+$. A full

Table 2. Quantification of HPB (4-hydroxy-1-(3-pyridyl)-1-butanone) derivatized with BSTFA (O-bis(trimethylsilyl)-trifluoroacetamide) in maternal and fetal cord blood samples using negative ion chemical ionization mass spectrometry.

Smoking status	Maternal (pmol HPB/g Hb)	Maternal (pmol HPB/g Hb)	Fetal (pmol HPB/ g Hb)	Fetal (pmol HPB/g Hb)
Non-smokers ($n=45$)	0.74±0.36	0.63±0.55	0.41±0.25	0.36±0.29
Passively exposed ($n=26$)	2.88±0.92	2.37±1.53	1.55±0.72	1.24±0.85
<1 pack per day smokers ($n=83$)	6.44±2.43	5.72±1.92	3.11±1.44	2.79±1.29
>1 pack per day smokers ($n=91$)	15.23±4.83	15.33±6.22	5.16±3.26	5.13±2.78

Table 3. Quantification of HPB (4-hydroxy-1-(3-pyridyl)-1-butanone) derivatized with BSTFA (O-bis(trimethylsilyl)-trifluoroacetamide) in maternal and fetal cord blood samples using electron ionization mass spectrometry.

Smoking status	Maternal (pmol HPB/g Hb)	Maternal (pmol HPB/g Hb)	Fetal (pmol HPB/ g Hb)	Fetal (pmol HPB/g Hb)
Non-smokers ($n=45$)	n.d.	n.d.	n.d.	n.d.
Passively Exposed ($n=26$)	1.66 ± 2.33	2.15 ± 2.04	1.86 ± 1.32	1.89 ± 1.42
<1 pack per day smokers ($n=83$)	9.22 ± 6.24	7.29 ± 3.71	4.64 ± 2.88	3.94 ± 2.36
>1 pack per day smokers ($n=91$)	1.94 ± 12.36	1.73 ± 9.34	1.16 ± 7.82	1.27 ± 10.4

n.d. not done.

scan m/z 50-450 PICI mass spectra for derivatized HPB by BSTFA was obtained by GC/MS and is illustrated in Figure 2, where molecular ion $[M+H]^+$ is 238 and m/z 222 corresponds to $[M\text{-}CH_3]^+$ whereas m/z 148 corresponds to the $[M\text{-}OSi(CH_3)_3]^+$ product.

NICI is a relatively soft ionization technique which produces parent molecular ions with relatively limited fragmentation of the parent molecule. As was similar with PICI, NICI produces low energy thermal electrons. Sample molecules absorb these thermal electrons and thus form negatively charged molecular ions M^-. NICI is more sensitive than PICI because of lack of formation of negative reagent gas ions. A full scan m/z 50-450 NICI mass spectra for derivatized HPB by BSTFA obtained by GC/MS is shown in Figure 3, illustrating the parent molecular ion of m/z 237 with limited fragmentary ions.

EI is a hard ionization technique compared to either PICI or NICI modes and this technique generates relatively small amounts of parent molecular ions but considerably larger quantities of molecular fragmentary ions. In this technique, the generation of both parent molecular ions and considerable fragmentary and patterns can be used to characterize not only chemical nature of the given unknown but also to some degree structural assignments. A full scan m/z 50-450 NICI mass spectra for derivatized HPB by BSTFA was obtained by GC/MS illustrating a molecular ion M^+ of m/z of 237 and fragmentary ions of a base peak of m/z 222 [M-CH3] and is shown in Figure 4.

Discussion

A comparative study for PICI, NICI and EI was performed to evaluate the suitability of GC/MS method for the detection of haemoglobin adducts to tobacco-specific nitrosamines in human samples. The results indicated that both NICI as well as PICI modes of detection provided greater sensitivity and detection of the released HBP from haemoglobin in both maternal and fetal samples. Both NICI as well as PICI techniques for it any cleaner resolution of the released adduct, making determination and quantitation easier as well as more convenient. Both of these ion techniques produce considerably less background noise as compared with electron impact modes of ionization and the signal-to-noise ratio in both maternal and fetal samples exceeded

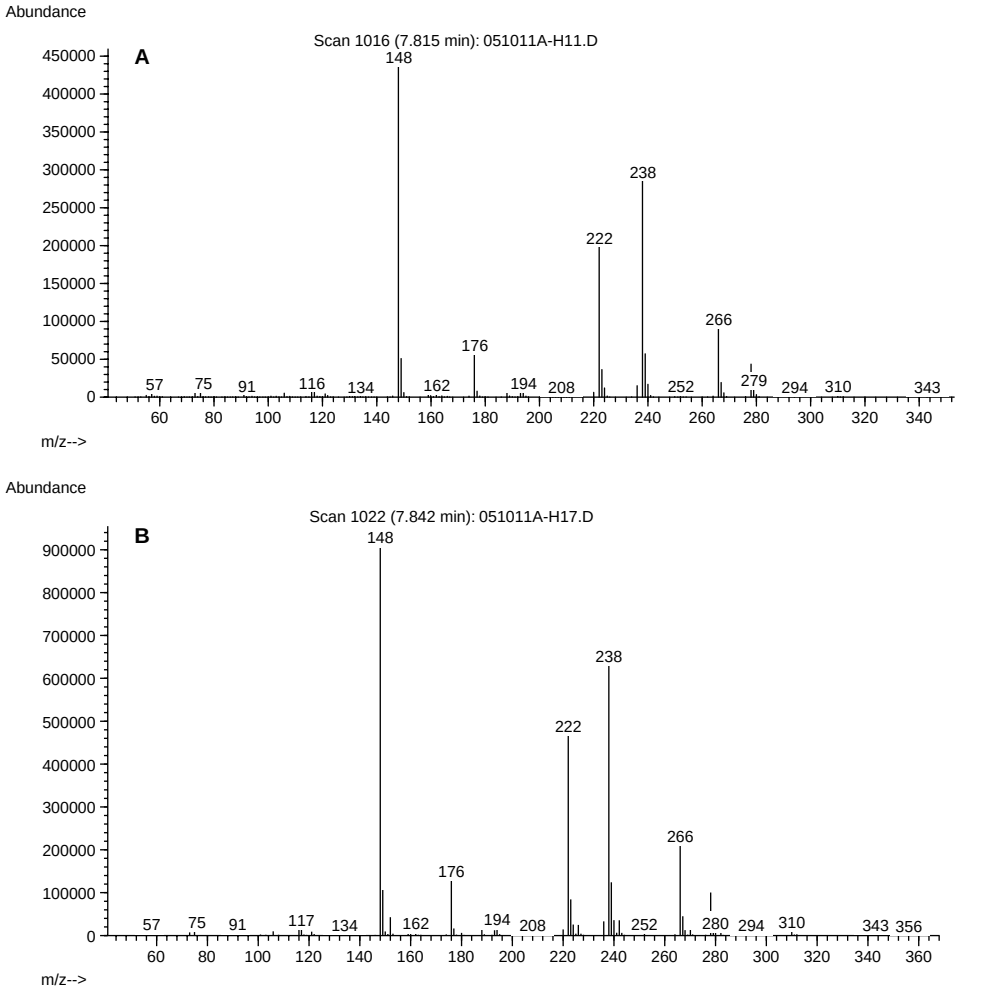

Figure 2. Positive ion chemical ionization spectra of trimethylsilyl derivatized 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) isolated from maternal smokers blood (>1 pack/day) (A) compared with the spectra of authentic derivatized HPB shown in (B).

20:1 in the chemical ionization modes compared to 3:1 in the electron impact ionization modes. Although both negative ionization as well as a positive ionization modes appear to be considerably more accurate and sensitive than the electron impact modes of ionization for the analysis of nitrosamine adducts of haemoglobin, the electron impact mode of ionization does afford benefits when compared to chemical ionization modes. Analysis of samples using electron impact mode of ionization allows the investigator to obtain not only parent molecular ions of both derivatized and non-derivatized samples, but also allows the obtaining of multiple fragmentary ions which can be used in structure Lu sedation and characterization of unknown adducts.

Both HPB and (3,3,4,4-D_4) HPB were derivatized by BSTFA and PFBC separately to improve the sensitivity of the compounds. These agents are well-established

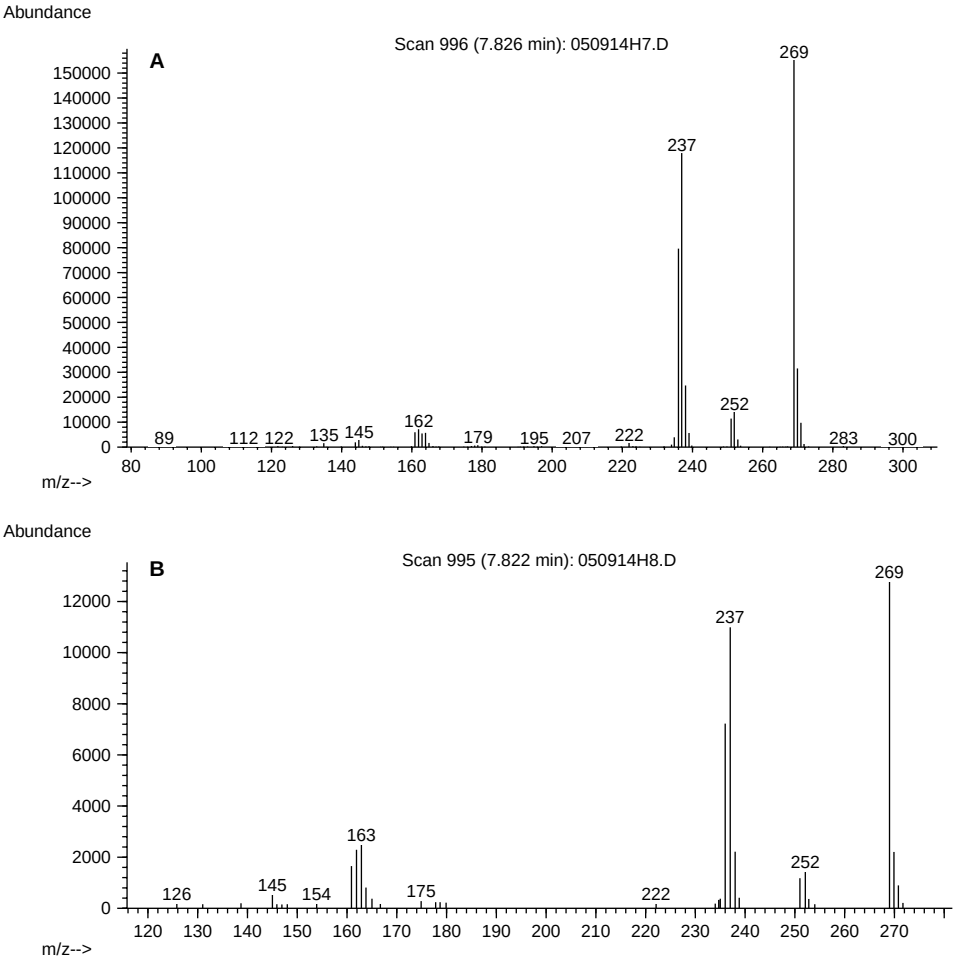

Figure 3. Negative ion chemical ionization spectra of trimethylsilyl derivatized 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) isolated from maternal smokers blood (>1 pack/day) (A) compared with the spectra of authentic derivatized HPB shown in (B).

chemicals used for enhancing detection of chemicals using gas chromatographic and mass spectral techniques and improve the detection sensitivity over the derivatized sample by approximately tenfold. In addition, the process of derivatization of the released HBP forms a highly volatile chemical that is easier to process and analyse using the described gas chromatographic techniques. Both derivatization techniques utilizing both BSTF and PFBC enhanced selectivity and sensitivity of detection when compared with non-derivatized samples. BSTFA derivatives are characterized as trimethylsilyl products of the hydroxyl group of HPB. On the other hand, derivatives performed with PFBC are characterized as a pentafluorobenzoate derivative of HPB. Comparative studies between the two derivatization techniques and among all three ionization techniques clearly showed that the PFBC derivatives of HBP resulted in a higher degree of sensitivity in the detection of both maternal and fetal nitrosamine–haemoglobin adducts. One drawback potentially to this type of derivatization is that the characterization of biological samples that are derivatized

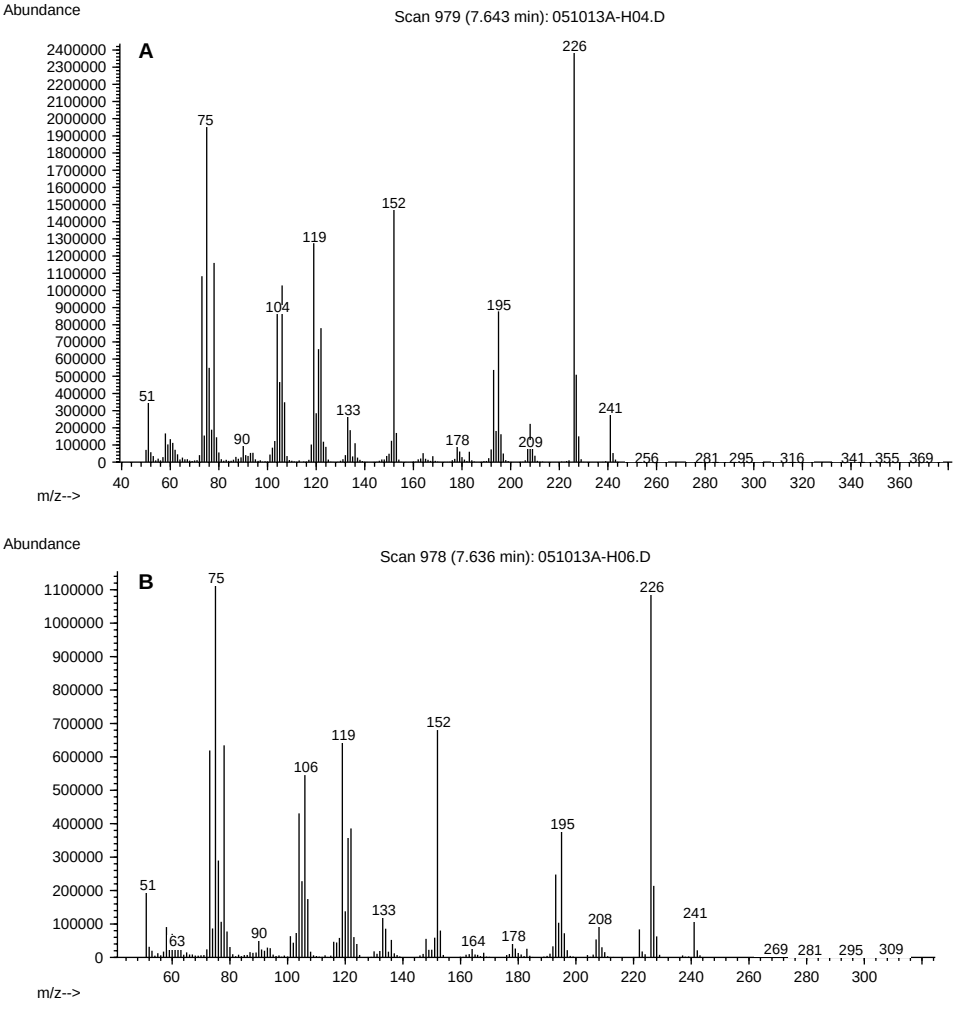

Figure 4. Electron impact ionization spectra of trimethylsilyl derivatized 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) isolated from maternal smokers blood (>1 pack/day) (A) compared with the spectra of authentic derivatized HPB shown in (B).

with PFBC results in more frequent cleaning of both injector ports on the gas chromatograph, as well as changing of the electron source. However, these trade-offs should not be diminished since there is increased sensitivity found in using this derivatizing agent. Furthermore, in the assessment of adducts in human samples one finds the necessity of having greater sensitivity of detection due to the low levels of exposure of some compounds.

This study demonstrates that upon a mild base hydrolysis HPB was released from lysed haemoglobin obtained from smoking and non-smoking mothers and fetal cord blood. In addition, increasing levels of both maternal and fetal tobacco-specific nitrosamines, as assessed via the detection of HBP released upon basic hydrolysis of haemoglobin, was found as smoking status increased from non-smokers to greater than one pack per day smokers. These studies indicate that mothers who smoke during pregnancy expose the unborn fetus to compounds found in tobacco, including

the tobacco-specific nitrosamines, many of which have been shown to cause animal tumours and teratogenesis. Although the matches to maternal and fetal sample pairs were obtained at the time of delivery, the study points to the fact that the placenta does allow the passing of carcinogens, such as those found in tobacco, into the fetus. While the study was limited to obtaining maternal and fetal samples at term, this does not preclude that damage can in fact occur to the developing fetus as a result of maternal smoking habits during pregnancy. During the latter months of pregnancy, and especially in the last trimester, fetal lung function is improved and the majority of weight gain of the fetus is achieved. Since it is known that exposure of animals to either tobacco smoke or to injection of nitrosamines leads to a variety of cancers, including lung cancer, it is not beyond the realm of possibility that exposure of the developing fetus to tobacco smoke could place the neonate at an elevated risk of the development of various cancers later in life.

Future studies will be directed at determining the efficacy of various modes of detection for environmental carcinogen adducts on haemoglobin, especially tobacco-related carcinogens, and the overall effect that these compounds have in the developing fetus. Specifically, we will be investigating early first-trimester exposure utilizing various biological markers such as haemoglobin adducts and amniotic fluid samples as indicators of exposure to harmful chemicals early in pregnancy. By understanding the relationship between exposure assessment, biological markers and risk of potential disease, we may be in a better position to both predict and prevent the potential carcinogenic effects associated with maternal smoking during pregnancy.

In these studies a new method was developed to quantify of nitrosoamine–haemoglobin adducts in mothers and cord blood samples. This method is very simple and less labour intensive. It also did not require further purification by HPLC and was ready to run by GC/MS. HBP was detected in the blood of mothers who smoked and cord blood at a low to high level depending on the smoking status of the mothers during their pregnancy. The level of HBP in cord blood was about half that in the respective mother's blood. No HBP was detected in the blood of non-smoking mothers and respective cord blood. PICI mode is more suitable than NICI and EI, to quantify samples at low level. BSTFA (N,O-bis(trimethylsilyl)-trifluoroacetamide) derivatization is more suitable than PFBC (2,3,4,5,6-pentafluorobenzoylchloride) to avoid frequent source cleaning. The newly develop method permits quantification of nitrosoamine–haemoglobin adducts in mothers and cord blood samples at a low detection limit. The detection limit of the instrument and the method are 30 and 50 fmol, respectively. This method could be used as a tool to measure biomarkers of tobacco exposure for smokers and non-smokers.

Previous studies determining smoking status in both non-pregnant subjects and individuals during pregnancy have been limited in the past to determining either urinary or serum levels of a specific metabolite of nicotine found in biological fluids, namely cotinine. Numerous assays and reports have been published over many years dealing with this biomarker of tobacco exposure and in large parts this is been an effective biological indicator of smoke exposure. However there are many limitations to the applicability and use of cotinine as a biomarker of tobacco exposure. One of the downsides of using cotinine as a specific biomarker of tobacco exposure is that the half-life of cotinine is relatively short in biological systems, approximately 24 h. The use of cotinine alone as a biomarker of tobacco exposure can lead to conflicting results when one takes into consideration the potential of both long and protracted labour in

childbirth. A more recent biological marker, such as that of using protein adducts of tobacco specific compounds provide a much clearer and much more precise assessment of current smoking status that is not interfered with by use of half-life of cotinine. In general sensitivities using both radio immunoassays as well as gas chromatographic methods for determining serum levels of cotinine from biological samples ranges in the low picograms per ml area. The sensitivities that are currently being reported utilizing protein adducts, especially haemoglobin adducts of tobacco-specific compounds, are comparable to the sensitivities at detecting smoke exposure using cotinine as a biomarker. Furthermore, the applicability of tobacco protein affects as indicators of smoke exposure allows us to gain a more time-weighted average of exposure assessment rather than a single point analysis as is reflected by cotinine determinations. The results contained in this paper clearly illustrate the applicability and use of haemoglobin as a biomarker of tobacco-specific nitrosamines found in tobacco smoke. In addition we have shown that the methods that are used allow both sensitive and accurate assessments of this biomarker in both maternal as well as in fetal blood samples taken at delivery. By assessing not only the maternal exposure during pregnancy but also fetal exposures taken at the time of delivery we may be in better position to assess the potential harm of these compounds during fetal gestation and delivery.

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